INVESTIGATION OF SMALL MOLECULE INHIBITORS FOR THE TREATMENT OF GLAUCOMA

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CERTIFICATE

I hereby declare that the short synopsis " *INVESTIGATION OF SMALL MOLECULE INHIBITORS FOR THE TREATMENT OF GLAUCOMA*" being submitted in partial fulfilment of the requirement for the degree of **Doctor of Philosophy in Biotechnology** under the Department of **Biotechnology** of the Manav Rachna International Institute of Research and Studies, Faridabad, during the academic year 2023. It is a bonafide record of original work carried out under the supervision of Dr. Rashmi Rameshwari, MRIIRS and Dr. Indrakant Kumar Singh, DU and has not been submitted in full or in part to any other university or institute for a degree or diploma. Further, it is certified that the short synopsis is plagiarism-free and the proof has been submitted.

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ABSTRACT

Glaucoma causes irreversible vision loss that has been diagnosed in more than 80 million individuals globally as of 2022, and by 2040, this figure is predicted to increase to 112 million. It is a degenerative condition that harms the optic nerve and impairs eyesight. With early diagnosis and treatment, it may be frequently avoided. This study will focus on treating glaucoma by targeting various pathways related to the disease. The conventional Rho kinase pathway has already been explored, and FDA-approved drugs for it. But then also, the side effects generated due to the prolonged usage of drugs persist, such as increased intraocular pressure and oxidative stress. Current therapies available in the market are primarily ROCK inhibitors or miotics. However, the current study will explore other pathways that support the *in vivo* and *in vitro* evidence. *In silico* methods, such as docking, ADMET analysis, MMGBSA analysis, and MD simulation of the shortlisted compounds, will be performed. This will aid in compound shortlisting as an alternative therapy to the convectional RHO kinase and ultimately result in promising compounds for glaucoma therapy.

After the *in silico* analysis, the top complexes will be examined on *in vitro* cell line to determine the effectiveness and potency of therapeutic molecules. Hence, this study aims to identify a therapeutic drug for treating glaucoma that decreases the risk of vision loss.

Keywords: Glaucoma, Ophthalmology, Inhibitors, Drug Discovery, Intraocular pressure, Aqueous Humor, Knowledge sharing

LIST OF ABBREVIATIONS

Abbreviations	Description
IOP	Intraocular pressure
AH	Aqueous humor
POAG	Primary open-angle glaucoma
PCAG	Primary closed-angle Glaucoma
TMW	Trabeculae mesh network
DKK1	Dickkopf-related protein
PDE	Phosphodiesterase
MMP	Matrix metalloproteinase

1.0 INTRODUCTION

Glaucoma is a chronic eye condition that causes permanent blindness worldwide, affects an estimated 88 million people worldwide, and is a leading cause of blindness. By 2040, the number of people affected by Glaucoma worldwide will rise to more than 112 million (Tham et al., 2014). Glaucoma is caused by increased eye pressure due to blocked fluid drainage in the eyes. This pressure harms the eye's optic nerve, resulting in vision loss and eventually blindness (Epstein et al., 1989; Sommer, 1991). It is often called the "silent thief of sight" as it is a slow-progressing eye disease that damages the optic nerve without noticeable symptoms.

Glaucoma can be treated with eye drops, laser surgery, or other medications. However, these treatments are often expensive and may not be effective for everyone. Therefore, pharmaceutical companies are researching new drugs to provide more effective treatments for Glaucoma. These drugs aim to reduce eye pressure, slow down the progression of the disease, and protect the optic nerve from further damage (Morrison, 2005; Rasmussen and Kaufman, 2005).

1.1 TYPES OF GLAUCOMA

Glaucoma can be classified into primary and secondary Glaucoma. Primary glaucoma can be classified further into Primary open-angle Glaucoma (POAG) and Primary angle-closure Glaucoma (PACG). Primary open-angle Glaucoma, the most prevalent type of Glaucoma, develops when the eye's drainage channels are clogged. This leads to a steady increase in pressure in the eye and can cause damage to the optic nerve (Mahabadi et al., 2022). POAG is most common in people over 40 and increases with age (Tham et al., 2014).

Open-angle Glaucoma is often painless, and its symptoms may not appear until the disease progresses. Angle-closure Glaucoma is less common but more serious. This happens when the iris is too near to the drainage angle in the eye, blocking the fluid and increasing eye pressure. PACG is most common in people over 60, and treatment involves medications to reduce eye pressure and surgery to open the drainage angle (Flores-Sánchez and Tatham, 2019). This form of Glaucoma can cause severe pain, vision loss, and even blindness if not treated quickly (Biswas, 2021). Primary open-angle glaucoma can be further classified into Juvenile glaucoma and Low-tension glaucoma (Dietze et al., 2023). Normal-tension Glaucoma is a kind of POAG that affects those with normal eye pressure and those with low blood pressure and heart issues. The other is congenital glaucoma, which affects one in every 10,000

infants. This can be cured if the newborns get surgery at a young age, which prevents irreversible vision loss (NIH, 2022).

Primary angle-closure glaucoma is divided into two types: acute and chronic angle-closure glaucoma. PAACG occurs when the angle between the eye and cornea is severely decreased, causing the IOP to rise. PCACG develops with age and remains for a long time; in this kind of glaucoma, the iris adheres to this angle, resulting in a permanent closure (Liu et al., 2021).

Researchers are working to develop new therapies and treatments that are more effective and have fewer side effects. The goal of glaucoma drug design is to develop drugs that can target specific areas of the eye, such as the trabecular meshwork, ciliary body, and sclera, which are involved in controlling IOP (Acott and Kelley, 2008; Martin et al., 2003). These drugs may reduce IOP and have fewer side effects than the currently available medications. Currently, drug design for Glaucoma is an active area of research. Researchers are exploring ways to use gene therapy and stem cells to treat Glaucoma. Gene therapy involves introducing a gene into cells to treat a disease. In Glaucoma, the goal is to reduce aqueous humor production, the fluid responsible for increasing IOP. Researchers are also exploring the use of stem cells to replace or regenerate cells damaged or lost due to Glaucoma (Quigley and Iglesia, 2004; Swetledge et al., 2021). In addition to drug design, researchers are also investigating ways to use nanotechnology to treat Glaucoma. Nanoparticles, too small to be seen with the naked eye, are being used to deliver drugs to specific eye areas (Davson et al., 2022). It allows for more targeted treatment of Glaucoma and reduces side effects.

1.2 HUMAN EYE STRUCTURE

1.2.1. The human eye is an incredibly complex organ made up of several distinct components that work together to allow us to see the world around us. The eye consists of the cornea, lens, iris, and retina at its most basic level. The cornea is a transparent layer of tissue that allows light to enter the eye and initiate the focusing process. Behind the cornea, a clear structure called the lens bends and focuses light to produce an image of the visual environment. The iris is the component of the eye that controls both the color and the amount of light passing through it. Last but not least, the retina is an inner layer of light-sensitive tissue that transforms light impulses into electrical messages that are sent to the brain and processed as a picture (Sunderland and Sapra, 2022). These components work together to provide us with vision.

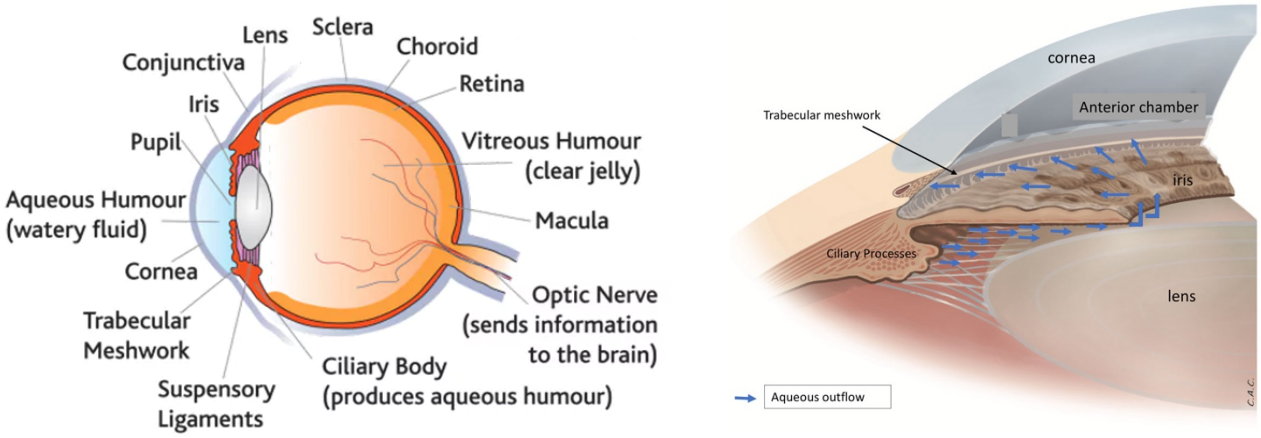

Figure 1: Illustration of an Eye displaying different components ("Secondary Glaucoma," n.d.) and the trabeculae mesh network drainage system for maintaining the flow of aqueous humor

(**Source**: https://www.glaucomapatients.org/basic/drainage-system).

1.2.2. Aqueous and vitreous humor are essential components of the eye which play a crucial role in its structure and functions. Aqueous humor is a transparent, watery fluid between the cornea and the lens. It helps to maintain intraocular pressure and provides nutrition to the lens and the corneal endothelium. It also helps to filter out dark spots or floaters in the visual field, providing overall clarity of vision.

Vitreous humor is a transparent, jelly-like fluid between the lens and the retina. It mainly comprises collagen and hyaluronic acid, helps maintain the eye's shape, and provides a cushion for the retina to rest on (le Goff and Bishop, 2008). It also helps to keep the lens in position and provides a shock absorber for the eye (Costandi Moheb, 2018; Sunderland and Sapra, 2022).

Both aqueous and vitreous humor is necessary for many of the functions of the eye, and both need to be balanced to ensure good vision. When these fluids are not in balance, it can lead to conditions such as Glaucoma, cataracts, and retinal detachment. It is essential to maintain the health of both the aqueous and vitreous humor to ensure clear and healthy vision.

1.2.3. The eye and brain connection is an integral part of the vision. The eyes are the windows to the

brain, and the brain processes the visual information received from the eyes. When we look at something, light is reflected off it, enters the eye, and focuses on the retina. The layer of light-sensitive tissue at the back of the eye is called the retina. The retinal cells convert light into electrical impulses, which are ultimately sent to the brain through the optic nerve (Gu et al., 2022). It is in the brain that the information is interpreted, and the image is processed.

The visual information sent to the brain is incredibly detailed. It includes information on the size, shape, color, and texture of an object, as well as its position concerning other things. This visual information is processed in different brain parts, including the visual cortex and the occipital lobe.

This connection between the brain and eyes requires the optic nerve, the nerve that connects the eye to the brain. Many retinal ganglion cell axons make up this structure, which transmits visual data from the eye to the brain. The central nervous system, which directs and coordinates bodily functions, includes the optic nerve. It is responsible for helping us interpret our visual environment and allowing us to recognize objects and movement. The retinal ganglion cells are in charge of conveying visual input to the brain. These cells are found in the ganglion cell layer, the retina's innermost layer. They transmit sensory information to the brain for processing and interpretation (R.F. Wang et al., 2015). The retinal ganglion cells and the optic nerve are essential components of the eye's structure and function. Without these components, people would be unable to see and interpret our visual environment. They are responsible for allowing us to analyse our environment and enable us to process and recognize visual information.

1.3. PATHWAYS ASSOCIATED WITH GLAUCOMA

1.3.1. Rho kinase (ROCK) is an enzyme that plays a crucial role in IOP regulation. ROCK1 and ROCK2 are two isozymes of the ROCK family that regulate ocular fluid dynamics. Their inhibition has been used to reduce IOP in animal models of Glaucoma (Sturdivant et al., 2016).

Recent studies have demonstrated that ROCK inhibitors can reduce IOP in glaucoma patients and improve vision in those with mild to moderate Glaucoma. Currently, there are only a few ROCK inhibitors approved by the FDA for glaucoma treatment, of which two are netarsudil (GS-7340) and ripasudil (KR-1214) (Garnock-Jones, 2014; Sturdivant et al., 2016). These drugs are administered as eye drops and are usually combined with other glaucoma medications, such as prostaglandin analogs, beta-blockers, and carbonic anhydrase inhibitors. ROCK inhibitors are safe and effective in reducing IOP in

glaucoma patients, and they have been shown to reduce the need for other glaucoma medications (Sotolongo et al., 2020). However, long-term safety and efficacy data are still needed to determine the best regimen for treating glaucoma patients. Furthermore, additional studies are required to evaluate the potential of ROCK inhibitors in combination with other glaucoma medications and examine the possible role of ROCK inhibition in treating glaucoma progression.

1.3.2. The **GSK-3 and Nitric oxide pathways** have been associated with the production of reactive oxygen species (ROS) and its relationship to Glaucoma. The GSK-3 is a class of enzymes involved in glucose metabolism, and its role in Glaucoma is thought to be related to its role in regulating the production of ROS, which leads to the damage of the optic nerve. Nitric oxide (NO) is another important molecule in the pathophysiology of Glaucoma, as it is suggested to play a role in regulating aqueous outflow. NO is involved in the production of ROS, leading to mitochondrial damage and increased production of ROS in Glaucoma (Miyazaki et al., 2010; Tezel, 2006).

1.3.3. High intraocular pressure (IOP) is one of the most common causes of Glaucoma. The **Calcium Calpain** pathway has recently emerged as an essential player in glaucoma pathogenesis. Calcium Calpain is a family of enzymes that can affect the activity of several cellular processes, including the regulation of IOP. In Glaucoma, Calpain-induced activation of the Rho/ROCK pathway has increased IOP (Huang et al., 2010). Recent research has explored the potential of Calpain inhibitors as potential therapeutic agents for Glaucoma. Some of these inhibitors are effective at reducing IOP in preclinical studies, while other inhibitors have been shown to reduce the pro-inflammatory activity of glaucoma-associated cells. These findings suggest that Calpain inhibitors may be helpful in treating Glaucoma, though further research is needed to fully understand their therapeutic efficacy (Tripathi et al., 1994).

1.3.4. TGFβ (transforming growth factor beta) is a protein that plays an essential role in the development and maintenance of the eye. TGFβ has been studied extensively in Glaucoma, as it helps to regulate the pressure inside the eye and influences the growth of new blood vessels. Recent research has found that TGFβ may also be involved in the development of Glaucoma by promoting the growth of the optic nerve cells. By regulating the proteins and pathways involved in the development of Glaucoma, TGFβ could be a potential target for new drug therapies. These therapies could be combined with existing treatments, such as laser surgery or medications, to reduce the risk of vision loss from Glaucoma (Chi et al., 2014). Further research is needed to determine the precise role of TGFβ in Glaucoma and the potential for new drug therapies targeting the protein.

1.3.5. Caspases- Apoptosis is started by the signaling molecule caspases. Inhibiting this pathway decreases glaucoma-related retinal damage because caspases produce IL-1, an inflammasome inhibitor that inhibits caspases and activates TLR, which can interact with NLRP1 and NLRP3 activator molecules. Researchers discovered that blocking TLR4 signaling directly reduces intraocular pressure (IOP) and retinal ganglion cell damage, providing a suitable signaling pathway to create a glaucoma treatment. In a nutshell, activating caspase8 and caspase1 by the ligand's binding to TLR4 leads to activating IL-1beta and other activator molecules such as NLPR3 and NLRP1 inflammasomes as well as ProIL-IBeta. This pathway, which provides a critical treatment strategy, is vital in controlling IOP and RGC degradation during Glaucoma (Chi et al., 2015; Kang et al., 2013).

1.3.6. MAP kinase- The MAP-kinase route is a pathway that TGF- beta activates. In Glaucoma, MAP kinase is critical in regulating the production of numerous proteins required for sustaining eye health. MMPs (matrix metalloproteases), proteins essential for maintaining adequate ocular pressure, are specifically produced. Additionally, MAP kinase promotes the activation of growth factors, which is necessary for the survival of retinal cells. The treatment approach for Glaucoma involves inhibiting MAP kinase to lower intraocular pressure. You can prevent MAP kinase from working by applying medication to the eyes. This approach results in lower levels of MMP and other protein production, which might elevate intraocular pressure. Additionally, it is thought to reduce the activation of growth factors which damage retinal cells (Alexander and Acott, 2003; Saccà et al., 2016).

1.3.7. Keap-nrf2-ARE pathway- The nuclear factor erythroid 2 and the Kelch-like ECH-Associating protein 1 are related factors. The 2-antioxidant response element pathway regulates a complete and efficient antioxidant response. It is essential for regulating inflammatory and oxidative stress responses inside an organism. This mechanism is controlled by the Keap1-Nrf2-ARE pathway, which also governs the Nrf2 response, inhibiting the antioxidant response. The retina layer, which is in direct contact with oxygenated tissue and has a high content of polyunsaturated fatty acids, is particularly impacted by ROS stress (Satoh and Lipton, 2017; Tezel, 2000). The trabecular mesh network regulates and drains the aqueous humor; thus, when it collapses, the aqueous humor flow imbalances, and IOP increases. When "8-hydroxy-2'-deoxyguanosine (8-OH-dG)" levels were high in glaucoma patients, the missing connection between ROS and TMW in Glaucoma was identified. This study discovered a statistically significant link between IOP and ROS. The TMW, retina, and optic nerves have been found to include hypoxia-induced factors and HSPs. ROS quantifies significantly high among the various targeted tissues (“Topical drug dosage forms for eye conditions,” 2017).

1.4. AVAILABLE TREATMENTS FOR GLAUCOMA

When it comes to treating Glaucoma, many different medications can be used to manage this condition. These medications come in a variety of dosage forms that are applied directly to the eye. The types of dosage forms that are used for glaucoma treatments include topical solutions, gels, suspensions, and eye drops. Each form has specific advantages and disadvantages that must be considered when determining which type of medication is best for an individual patient's needs. Topical solutions are the most common dosage form used for glaucoma treatments. These medications are designed to absorb by the eye quickly and are typically applied twice daily. Topical solutions effectively lower the intraocular pressure in the eye, which is the leading cause of Glaucoma ("Topical drug dosage forms for eye conditions," 2017). However, they can also cause side effects such as burning, stinging, and dryness of the eyes. Gels and suspensions are also commonly used dosage forms for glaucoma treatments. These medications are designed to be slow-release and are applied directly to the eye once every two weeks. Gels and suspensions are effective for controlling the pressure in the eye, but they can cause discomfort and irritation to the eye (Harvard Eye Associates, 2022).

Eye drops are another type of dosage form for glaucoma treatments. They can be used either as a monotherapy or in combination with other medications. Eye drops are effective for controlling the eye's intraocular pressure but can cause stinging, redness, and irritation when applied.

In some cases, a laser procedure called selective laser trabeculoplasty (SLT) may be used to help the fluid pass more freely. During the SLT procedure, a narrow beam of high-energy light opens up blocked areas in the trabecular meshwork to help the fluid flow more freely out of the eye (Aref et al., 2013). Another option for treating Glaucoma is to surgically implant a drainage device, such as a glaucoma drainage implant (GDI). The GDI is a tiny tube inserted into the eye and helps the fluid drain out of the eye, reducing the pressure and helping to preserve eye health (Zhou et al., 2020). Stem cell therapy is innovative in treating many diseases, including Glaucoma. Scientists have shown that stem cell therapy may be an effective treatment for Glaucoma. In recent years, numerous studies have suggested the potential benefits of stem cell therapy for a wide range of conditions, including Glaucoma.

A study published in 2015 found that autologous adipose-derived stem cells (ADSCs) can reduce IOP and improve visual acuity in patients with primary open-angle Glaucoma. Another study found that ADSCs can promote healing and minimize scarring after cataract removal surgery in people with

Glaucoma (Lambuk et al., 2022). Nanoparticles with anti-inflammatory properties have also been shown to reduce IOP in experimental models of Glaucoma (Hua et al., 2020). Other studies have shown that transplantation of embryonic stem cells (ESCs) into the anterior chamber of the eye can prevent the progression of retinal damage and vision loss associated with Glaucoma (Yang et al., 2021).

1.5. CHALLENGES

Existing treatments often need to be combined with other drugs. This can make them difficult to adhere to and lead to poor outcomes. Developing drugs that work alone or in combination with other treatments would be an essential step forward in treating Glaucoma.

Other challenges include lack of efficacy, safety, availability, and affordability, poor adherence and compliance by patients to long-term regimens of medications, and limited therapeutic options. Many modern drugs for Glaucoma target the overproduction of aqueous humor or ocular hypertension. However, when these agents no longer work adequately, the result is often drug intolerance and increased costs. Therefore, there is a critical need for new and more effective therapies to treat this condition.

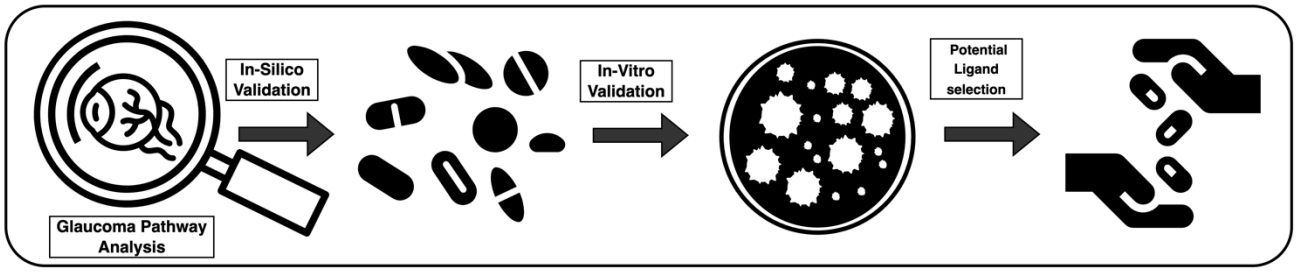

Figure 2: Proposed methodology for identifying potential ligand molecules to treat Glaucoma**.**

The current study will explore pathways other than the conventional RHO kinase pathway that are significant to glaucoma and have not been extensively explored by *in vitro* and *in silico* analysis. In the present study, work will be done on identifying the compounds that will decrease intraocular pressure and the oxidative stress generated during glaucoma. And finding a better compound that will have fewer side effects is the aim of this study.

2. LITERATURE REVIEW

Glaucoma was identified in 1851; Heinrich Mueller was the first to visualise the depression in the optic disc. From then on, people started researching and exploring the treatment for glaucoma. In 1996, the first FDA-approved drug for glaucoma was developed, and till now, many studies have been conducted, which will be discussed in this section.

According to a study conducted by Jiawei Yang. in 2021, it was found that the baicalein therapy lowered Intraocular pressure and reduced myelin sheath and axonal damage. Reduced apoptosis in the ganglionic cell line may be connected to the therapeutic benefits of baicalein on Glaucoma (Yang et al., 2021). Vasudha Abbhi. in 2017, identified that another chemical, Benzimidazole derivatives at 0.5- and 1-mM concentrations, the nitrophenyl piperazine substituted drug 9j significantly reduced IOP (51.56%) and inhibited ROCK II enzyme by 57.25 and 77.92%, respectively. It also showed a significant amount of free radical foraging activity with an IC50 value of 95.49 mg/mL in the DPPH test. Compound 9j is a promising antiglaucoma drug since molecular docking studies have shown that it binds to the active site of recombinant human ROCK II (Abbhi et al., 2017). Another compound, ST075640, identified in the Hemavathy Nagarajan study, modified both ocular blood flow and IOP in glaucoma and was determined to be a potential target for both receptors (ETA and ETB). This would stop the depletion of Retinal Ganglion Cells. The efficiency of the found hit may also be determined by utilising “in vitro collagen gel contraction and myosin light chain phosphorylation” tests; this function verifies the ST075640's prospective target for testing in vitro to cure Glaucoma (Nagarajan and Vetrivel, 2021).

Compared to the rho GTPase-mitomycin complex, “the rho GTPase-delphinidin complex” was more stable regarding energy. Comparative examination of the two chosen compounds (Cyanidin and Delphinidin) was performed using “RMSF, RMSD, RG, SASA and PCA”. The investigation was done by Sumit Kumar Rai. 2021 showed that delphinidine is more effective than cyanidin and other commercially available conventional medicines for inhibiting Rho GTPase in terms of binding free energy and stability. This compound has strong pharmacodynamic and pharmacokinetic properties, proving it to be a potential compound (Rai et al., 2021).

It is know that apoptosis of retinal ganglion cells (RGCs) is the main pathophysiology of glaucoma, and mitochondrial dysfunction is a significant pathogenic component. In a docking study by Akiko Miki 2014, syntaphilin was found to affect “mitochondrial mobility”. However, few studies have examined the function of “syntaphilin” in the optic system. It examined how syntaphilin was expressed in the rat brain, ONH, and retina. The visual system displays a variety of syntaphilin expression patterns.

Syntaphilin was found in the rat ONH, retina, and neuronal cell bodies astrocytes in the superior colliculus. Its expression in the ONH reduced between days 3 and 7. This demonstrates that axonal damage may be caused by syntaphillin expression in the optic nerve astrocytes (Miki et al., 2014).

In a recent study Ji-Hong Wu. 2013 found that increased IOP can cause Drp1 to be upregulated and mitochondria to undergo fission, both of which contribute to apoptosis. The mechanism underlying these effects is still unclear; however, this study examined 24 kinases linked to mitochondrial functions for their impact on hydrostatic pressure-induced DRP1 expression using RNAi screening. For the first time, the researcher found that GSK3 knockdown inhibited the increase in DRP1 by increasing pressure. This demonstrates the crucial role GSK-3 plays in glaucoma (Wu et al., 2013).

Another disease, the hereditary retinal disease known as Retinitis Pigmentosa (RP), is now both incurable and untreatable. As the retina is a component of the CNS, "glycogen synthase kinase 3" (GSK-3) inhibitors are prospective strategies for retinal cell neuroprotection. GSK-3 inhibitors are powerful neuroprotective agents. To confirm and validate the use of GSK-3 as a pharmaceutical target, Miguel Marchena. 2017 carried out a study in which a variety of small compounds with various potencies and binding modalities for GSK-3 were tested. Similarly, when Glaucoma is present, protecting retinal cells is necessary; thus, tracking pertinent information related to retinal cell protection (Marchena et al., 2017).

"Secreted frizzled-related protein-1 (sFRP-1), an inhibitor" of Wnt signaling, plays a unique role in controlling IOP. In "glaucoma HTM cells", sFRP1 was overexpressed in genes contributing to the Wnt pathway cascade as coded by Wan-Heng Wang et al. 2008. According to these findings, in glaucoma, excessive IOP is considered to be generated by increased sFRP1 expression in the TM, which restores "Wnt "signaling in the TM" and maybe a potential disorder treatment method for glaucoma" (Wang et al., 2008).

Another compound theophylline reduces MMP activity and aqueous outflow, while sildenafil inhibits PDE5, increasing cGMP, vasodilation, and blood flow away from the ocular circulation. This *in vitro* study of Stephanie P. Chen. 2017 further supports the relationship between PDEi use and the development of Glaucoma (Chen et al., 2017). AA or A-A were applied topically to the eyes, and the higher IOP induced by PDE6D overexpression in mice eyes were reversed. The discovery that PDE6D is the molecular partner of AA sheds light on the potential use of this therapeutic candidate in the management of Glaucoma as per the study conducted by Allan R Shepard et al. 2013. (Shepard et al., 2013). B Tian. In 1998 found that when the serine-threonine kinase inhibitor, H-7, was applied to bovine aortic endothelial cells, actin microfilaments were irreversibly disrupted in a concentration and time.

Cell-cell and cell-matrix adhesion was also altered, and a decrease in intraocular pressure was observed. This demonstrates how H-7 affects actin microfilaments and aids in glaucoma therapy (Tian, 1998).

According to the study performed by Ahnul Ha. 2022 the Sovesudil, a ROCK inhibitor that primarily targets TMW, had a statistically significant IOP-lowering impact at both 0.25% and 0.5% TID; upon treatment, the 0.5% dosage met the criteria for superiority vs the placebo. Individuals with normal-tension Glaucoma tolerated sovesudil well. Only minimal side effects were observed during therapy, such as a very infrequent occurrence of conjunctival hyperaemia (Ha et al., 2022).

In the TM cells of people with Glaucoma, sFRP1 is overexpressed. In *ex vivo* perfusion-cultured human eyes, the addition of recombinant sFRP-1 reduced outflow, followed by lower levels of β-catenin, the Wnt signaling mediator, in the TM. IOP was considerably lowered by topical ocular administration of an inhibitor of a downstream suppressor of Wnt signaling after vector injection, which caused a two-fold rise. According to the findings of Wan-Heng Wang. 2008, it was found that the raised IOP in Glaucoma is thought to be caused by increased sFRP1 expression in the TM. Restoring Wnt signaling in the TM may be a potential disease treatment technique for treating Glaucoma (Wang et al., 2008).

In patients with primary open-angle Glaucoma, a combination treatment of carteolol/latanoprost (LCFC) and timolol/latanoprost (LTFC) was investigated. Increased IOP in the two groups decreased by 0.91 mmHg and 0.68 mmHg, respectively. The LCFC group's night pulse rate rose, whereas the LTFC group was consistently lower. Patients in the LCFC group had 24-hour IOP curves comparable to those in the LTFC group. However, based on the findings of Yuta Saito. in 2021 regarding pulse rate, 1st group had a less significant 24-hour impact on the cardiovascular system than 2nd group (Saito et al., 2021).

Also, Brimonidine/timolol fixed-combination (BTFC) ophthalmic solutions with and without preservatives were evaluated in a study by Joon Mo Kim. 2021. The percentage of patients in the PF group who experienced IOP reductions of between 15% and 20% and IOP at all times were not substantially different from those in the PC group. Regarding the frequency of adverse events, there were no evident variations between the two groups. Research demonstrated that PF and PC BTFC solutions work identically (Kim et al., 2021).

In a study by Ahmed Essam Faseeh. 2021 the tests were conducted to identify the variations between latanoprost, travoprost, and tafluprost. It was found that the IOP between the three groups during the different time periods did not significantly differ. At the follow-up visits, similar non-significant changes were observed. Tafluprost substantially reduced IOP in this group ($p = 0.03$). All three drugs significantly

and equally reduced IOP and IOP fluctuations, revealing their equivalent tolerability with only minor variations in their adverse effects (Faseeh et al., 2021).

In 2018 Kyoung In Jung. using the information from participants under the age of 40 from the Korean National Health and Nutrition Examination, created a survey to determine the relationship between dietary nutrient consumption and Glaucoma. Using the 24-hour recall approach, the amount of fibre, ash, calcium, phosphorus, iron, salt, potassium, retinol, vitamin A, thiamine, riboflavin, niacin, and vitamin C consumed was calculated. In a multivariate analysis, only niacin was linked to Glaucoma in patients with IOP 21 mmHg ($p = 0.022$). This demonstrated that dietary nutrient consumption was linked to open-angle Glaucoma independent of IOP. Niacin consumption among all nutrients was lower in those with normal tension glaucoma (Jung et al., 2018).

Yuichi Ikeda. 2004 identified that Vasorin is a transmembrane glycoprotein that is widely distributed in smooth muscles that interact with TGF to stop TGF cellular activity. Some metabolic and biological processes it supports include cell division, differentiation, motility, angiogenesis, and calcification. Vasorin is mainly found in aqueous humor secretions and TMW cells. Vasorin demonstrates anti-TGF activity and improves TMW cell regulation (Ikeda et al., 2004).

Another distinct natural chemical called ligostrazine (TMP) regulates the PI3K-Akt/mammalian target of the rapamycin (mTOR) signaling pathway by interfering with phosphatidylinositol 3-kinase (PI3K), and protein kinase B (Akt) was identified by Shiyong Wu. 2018. Ligustrazine performs several different biological tasks in cells, such as suppressing autophagy and apoptosis in retinal cells, yet it does not affect IOP levels. This is a crucial target molecule that can be employed to defend cells against glaucomatous conditions (Wu et al., 2018).

Takeru Ehara. 2018 explored Nitric oxide (NO) as an endogenous receptor for soluble guanylate cyclase (sGC), which has been linked to several illnesses brought on by oxidative stress. A malfunctioning sGC/NO/cGMP pathway has been reported to play a role in the increased intraocular pressure linked to Glaucoma. We learned about identifying compounds that may activate oxidised sGC and restore its catalytic activity for synthesising cGMP when applied topically to the eyes. This led to the identification of compound (+)-23, which upon application, significantly decreases intraocular pressure in a cynomolgus model (Ehara et al., 2018).

The pathogenesis of Glaucoma is linked to the Rho-associated protein kinases (ROCKs). In recent years, the discovery of ROCK inhibitors has received much interest. A novel family of ROCK inhibitors known as 3,4-dihydrobenzo[f] [1,4]oxazepin-5(2H)-one derivative has been identified by Yumeng Sun. 2021.

In an ocular normotensive model, the mean IOP-lowering impact of 12b was 34.3%, and no overt hyperemia was noticed. Compounds were shown to have strong anti-ROCK I and ROCK II actions, with IC50 values of 93 nM and 3 nM, respectively (Sun et al., 2021).

Monika Lakk. 2021 explored that cholesterol raises the likelihood of the occurrence of pressure-related disorders like Glaucoma. The stretch-activated transient receptor potential vanilloid isoform 4 (TRPV4) channels in human trabecular meshwork (TM) cells were examined about mechanical strain, free membrane cholesterol, the actin cytoskeleton, and other factors. It was discovered that the TRPV4 channels are primarily present outside the domains of the lipid membrane that are rich in cholesterol and aid in the maintenance of the shape of the membrane. In Glaucoma, this modifies the traditional outflow pattern and intraocular pressure providing a potent pathway to treat Glaucoma (Lakk et al., 2021).

Another aspect of treating glaucoma is that Actin cytoskeletal remodelling and ECM deposition are caused by TGF signaling in numerous tissues. This work by Alexander Kirschner in 2021 suggests mechanosensitive channel blockage as a promising strategy to alter ONH astrocyte behaviour in Glaucoma. According to research, mechanosensitive channel blocking improves reactive gliosis and actin cytoskeletal dysregulation in glaucomatous astrocytes. Changes in astrocyte behaviour have an impact on ganglion cell function since they are the primary support cell for retinal ganglion cells. The levels of GFAP, fibronectin, and collagen IV were considerably raised by TGF2 treatment, while the immunoreactivity of GFAP was decreased by GsMTx4 co-treatment. According to the study, inhibiting the activity of mechanosensitive channels can be used as a treatment for Glaucoma to control the actin cytoskeletal remodelling of the optic nerve head (Kirschner et al., 2021).

All of the study's findings were highly helpful in identifying the gaps in developing a treatment for glaucoma.

3. GAPS IDENTIFIED

Developing novel treatments for Glaucoma has been a challenge for many years. Still, with few new drugs being developed in recent decades, no complete cure is available. One major problem faced during drug development for Glaucoma is that it is difficult to target the disease-causing cells in the eye. This makes it difficult to create drugs that specifically target the cells that cause Glaucoma, resulting in drugs that may have unwanted side effects. Based on the literature survey undertaken, the following gaps have been identified:

1. After exploring a huge amount of literature, it was identified that many pathways play a role in glaucoma. But significant potential pathways were identified for Glaucoma, and it was found that the Rho-kinase pathway is the primary target of several drugs available today. In silico research should focus on pathways including Nitric Oxide Guanylate, MAP Kinase and GSK3Beta because GSK3 and NO's mediator PDE has a strong link with Glaucoma. This study is optimistic about developing a better medication that targets alternative pathways. By focusing on and targeting these pathways, Glaucoma can be controlled as these pathways have potent in *vitro* and *in vivo* evidence.
2. In addition to targeting IOP and aqueous humor flow, which is the gold standard for Glaucoma drugs, other factors such as TMW maintenance, ROS generation, RGC survival, optic nerve injury, and actin cytoskeleton flexibility may also be targeted.
3. Most medications in the market are prostaglandin analogs; and ROCK Inhibitors or miotics, but based on the evidence, other protein targets will be explored.

Therefore, in this study, as quoted above, pathways other than RHO Kinase will be focused on (Gap 1). This will be undertaken to identify a potential target other than the conventional RHO kinase so that it can be utilized to find a novel inhibitor for the treatment of Glaucoma.

4. OBJECTIVE OF RESEARCH

The objectives of the proposed study are

I. To identify dysregulated pathways and druggable target involved in Glaucoma from the literature, supported by *in vitro* evidence.

II. To target selected protein *in silico* by docking, ADMET and pre-simulation MM-GBSA analysis.

III. To shortlist compounds using MD simulation, post-simulation MMGBSA analysis and manual visualization.

IV. To evaluate top-hit compound *in vitro* on a glaucoma cell line using MTS viability, TUNEL and DPPH assays.

5. PROPOSED METHODOLOGY OF THE STUDY

The proposed study will be carried out accordingly:

1. **Identification and selection of dysregulated pathways**

   A thorough literature survey will be carried out to identify the pathways associated with Glaucoma. Shortlisting of a target will be done based on the literature having wet lab data supporting its significant role in Glaucoma.

2. **Selection of drug target and preparation of compound libraries**

   The selected drug target will be used for further study. The commercially available drugs database, such as Asinex, Selleckchem, Enamine etc., will be used to prepare the ligand library.

3. ***In silico* preparation of protein and grid generation**

   The inhibitor-bound protein and the ligand library will be prepared using the Schrodinger tool. Based on the protein PDB the grid will be prepared to fix the docking site.

4. **Docking, ADMET, and pre-simulation MM-GBSA analysis**

   HTVS docking followed by XP docking will be performed. Compounds will be shortlisted based on the docking pose and docking score. The shortlisted compounds will be used to calculate the binding free energy. Further, compounds having the highest negative MMGBSA values compared to the reference will be analyzed further by MD simulation.

5. **Molecular dynamic simulation studies, post-simulation MM-GBSA analysis and manual visualization**

   MD simulation for 100 ns will check the stable interactions between the protein and the ligand during the simulation. Compounds with the least fluctuations and stable binding interactions will be further considered for post-simulation MMGBSA analysis. Pre- and post-MMGBSA energies will be compared to select the final compounds for in-vitro studies.

6. **Check the survivability of cells using the MTS-IC_{50} and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**.

   The effectiveness and potency of the shortlisted leads will be investigated on the Glaucoma cell lines. This will involve the protection of retinal cells from cell death and the dose concentration required to deliver the desired effect. The TUNEL assay will determine whether there is a decrease in cell death and an increase in cell survival.

7. **Check the reduced oxidative stress of cells by α, α-diphenyl-β-picrylhydrazyl (DPPH) assay**

   As ROS plays a vital role in RGC apoptosis, the effect of the treatment on scavenging ROS generation and the drug's efficacy in lowering ROS formation will be assessed.

6. TIMELINE

Objectives		2nd Year																						3rd Year											
		Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
	PhD Coursework																																		
In-Silico	Library and protein preparation																																		
	Library Filteration, Docking and ADMET																																		
	MMGBSA and MD Simulation																																		
	Post MMGBSA																																		
	Meta Dynamics																																		
	Shortlisting of Compounds																																		
In-Vitro	Cell line maintainance																																		
	Cytotoxicity assays (MTS)																																		
	TUNEL Assay																																		
	Kinase activity and apoptosis assay																																		
	DPPH Assay																																		
	Publication																																		
Other	Patent																																		
	Further Progress																																		

7. EXPECTED OUTCOME OF THE STUDY

This planned research aims to uncover and characterize novel compounds for glaucoma treatment. The proposed lead is expected to bind to the selected protein's site of action so the vision-threatening functions can be controlled. This will open up new avenues for glaucoma therapy and better strategies that may be more effective in eradicating the disease. For people with the illness, the future of glaucoma therapy is very exciting. Research is still being done to create more efficient, available, and inexpensive therapies. Some of the most promising developments are gene therapy, combination treatments to lower glaucoma-related intraocular pressure, and the creation of novel medications that can target and block the growth of new capillaries in the eye that contribute to glaucoma progression. Exploring pathways other than Rho-kinase and finding a potential compound that binds to the target site for a long duration will be the focus of this study. Glaucoma therapy has a bright future, and researchers are working hard to make it a reality.

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INVESTIGATION OF SMALL MOLECULE INHIBITORS FOR THE TREATMENT OF GLAUCOMA

by Vanshika Rustagi

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INVESTIGATION OF SMALL MOLECULE INHIBITORS FOR THE TREATMENT OF GLAUCOMA

SHORT SYNOPSIS

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ABSTRACT

Glaucoma causes irreversible vision loss that has been diagnosed in more than 80 million individuals globally as of 2022, and by 2040, this figure is predicted to increase to 112 million. It is a degenerative condition that harms the optic nerve and impairs eyesight. With early diagnosis and treatment, it may be frequently avoided. This study will focus on treating glaucoma by targeting various pathways related to the disease. The conventional Rho kinase pathway has already been explored, and FDA-approved drugs for it. But then also, the side effects generated due to the prolonged usage of drugs persist, such as increased intraocular pressure and oxidative stress. Current therapies available in the

market are primarily ROCK inhibitors or miotics. However, the current study will explore other pathways that support the *in vivo* and *in vitro* evidence. *In silico* methods, such as docking, ADMET analysis, MMGBSA analysis, and MD simulation of the shortlisted compounds, will be performed. This will aid in compound shortlisting as an alternative therapy to the convectional RHO kinase and ultimately result in promising compounds for glaucoma therapy.

After the *in silico* analysis, the top complexes will be examined on *in vitro* cell line to determine the effectiveness and potency of therapeutic molecules. Hence, this study aims to identify a therapeutic drug for treating glaucoma that decreases the risk of vision loss.

Keywords: Glaucoma, Ophthalmology, Inhibitors, Drug Discovery, Intraocular pressure, Aqueous Humor, Knowledge sharing

1.0 INTRODUCTION

Glaucoma is a chronic eye condition that causes permanent blindness worldwide, affects an estimated

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